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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The goal of this research was to demonstrate the feasibility of the dog model of spontaneous prostate carcinogenesis as a valuable animal model system to evaluate potential chemopreventive agents. Specifically, we sought to determine if androgen deprivation, or supplementation with dehydroepiandrosterone (DHEA) or selenium modulates biomarkers of prostate carcinogenesis in elderly male dogs. To accomplish this, we conducted a 6 month laboratory intervention trial using 49 purchased elderly intact male beagle dogs randomly assigned to 1 of 5 treatment groups: (1) no treatment; (2) 3 μg/kg selenomethionine PO; (3) 6 μg/kg selenomethionine PO; (4) DHEA 100mg/kg PO; or (5) surgical castration. Although our final data analysis is not complete, our findings show that daily selenomethionine supplementation for 6 months in elderly, sexually intact beagle dogs (physiologically equivalent to 62-67 year-old men) results in a significant reduction in DNA damage within the prostate. This effect on the aged prostate occurs without upregulation of the serum activity of the antioxidant enzyme, glutathione peroxidase. Interestingly, DHEA supplementation exerts a DNA damage-sparing effect within the aged prostate despite increased serum androgen concentration. Our results challenge existing paradigms regarding the "antioxidant" chemopreventive effect of selenium and the potential hazards of DHEA. We intend to further develop this unique animal model system using our Phase II award.

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INTRODUCTION

Dogs and humans share a vulnerability for the spontaneous development of prostate carcinoma. Prevention rather than treatment may be the best approach to reduce the morbidity and mortality associated with prostate cancer. Our previous work documented the high prevalence of high grade prostatic intraepithelial neoplasia (HGPIN) in elderly pet dogs and its close association with invasive carcinoma. *In vivo* screening of promising chemopreventive agents using the dog model of spontaneous prostate carcinogenesis represents a novel approach to the prevention of prostate cancer. In particular, use of pet dogs that share the same environment as humans allows the simultaneous investigation of risk factors and biomarkers for prostate carcinogenesis in an outbred population. The studies proposed herein represent our first attempt to exploit the canine model. In this Phase I award, our first task was to conduct a short-term (6 month) study with retired breeder dogs to determine if androgen deprivation or supplementation with the controversial androgenic steroid dehydroepiandrosterone (DHEA) or selenium modulates DNA damage or other biomarkers of prostate carcinogenesis. Our second task was to submit a proposal for Phase II funding to study further the effect of selenium supplementation on molecular biomarkers of carcinogenesis in the aged dog prostate. The long-term objective of this research is to utilize the dog as a pre-clinical model to test innovative ideas in cancer prevention and treatment, as well as to further understand the factors that regulate prostate cancer development and progression.

BODY

Task 1: To determine if androgen deprivation, or supplementation with DHEA or selenium modulates biomarkers of prostate carcinogenesis

To accomplish this, we conducted a 6 month laboratory intervention trial using 49 purchased elderly intact male beagle dogs. After prostatic biopsy, dogs were randomly assigned to 1 of 5 treatment groups: (1) no therapy (control) (n = 10 dogs); (2) 3 μg/kg selenomethionine PO (n = 10 dogs); (3) 6 μg/kg selenomethionine PO (n =10 dogs); (4) DHEA 100mg/kg PO (n = 10 dogs); or (5) surgical castration (n= 9 dogs). Interventions were well tolerated by all dogs. Although the final data analysis is not yet completed, our project has generated exciting new information on the possible mechanisms of 2 potential prostate cancer preventing agents: selenomethionine and DHEA. Our findings are summarized below:

EFFECT OF SELENOMETHIONINE ON MOLECULAR BIOMARKERS OF CARCINOGENESIS WITHIN THE AGED DOG PROSTATE

A. Selenium Supplementation Results in a Significant Reduction in Accumulation of DNA Damage Within the Aged Dog Prostate **(Table 1)**

We used single cell gel electrophoresis (comet assay) to measure the effect of selenium supplementation on DNA damage within the prostate. The alkaline comet assay (single cell gel electrophoresis) is a robust and versatile assay used to measure extent of DNA damage on a single cell basis [Singh et al. Exp Cell Res 175: 184 (1988)]. With the assistance of Dr. Raymond Tice, an expert in comet assay, we have adapted comet to assess DNA damage

within prostatic epithelial cells in prostate tissue harvested at autopsy or from a single 18 gauge needle biopsy. Daily selenomethionine supplementation for six months in elderly, sexually intact beagle dogs (physiological age equivalent to 62-67 year old men) resulted in a significant 33% reduction in DNA damage within the prostate. Selenomethionine at 3μg/kg/day and 6μg/kg/day had similar DNA damage-sparing effects within the prostate. Our results show that six months of supplementation with a non-toxic dose of selenomethionine (3 μg/kg/day is comparable to the 200μg daily dose that will be used in the SELECT trial) decreases the accumulation of DNA damage in the prostate of elderly dogs.

B. DNA Damage-Sparing Effect of Selenomethionine in the Aged Prostate Occurs Without Upregulation of Serum Activity of the Antioxidant Enzyme Glutathione Peroxidase **(Table 1)**

In our experiments, elderly dogs were fed a selenium adequate diet (0.3 ppm as is basis). This enabled us to study the mechanisms of selenium supplementation in a model of selenium adequacy, which is the case in almost all healthy Americans, including those who will participate in the PCPT-2 SELECT trial. These data are consistent with the Nutritional Cancer Prevention Trial of Clark et al. [J Am Med Assoc 276:1957 (1996)] in which prostate cancer incidence in the selenium supplemented group was decreased by 60% despite no change in serum glutathione peroxidase activity (unpublished data from GF Combs, CoInvestigator Phase II project). These data are intriguing because they suggest that *selenium supplementation may exert its prostate cancer protective effects by a mechanism independent of its antioxidant properties.*

C. Selenium Supplementation May Decrease DNA Damage Within the Prostate by Upregulation of Apoptosis

Our preliminary data indicate a very low level of apoptosis within the prostate epithelial cells of intact control dogs. In contrast, there was an almost 10X increase in mean number of apoptotic cells per field in dogs supplemented with 6μg/kg/day selenomethionine ($p=0.12$). Within the selenium supplemented dogs, there were markedly upregulated foci of apoptosis, defined as fields in which the number of apoptotic cells per field was > 20x the average for control dogs. These foci were never found in intact control dogs. *The possibility that selenium may exert its DNA damage sparing effect by upregulation the programmed cell death of DNA damaged epithelial cells within the prostate is a novel hypothesis that deserves further study.*

D. Selenomethionine Supplementation is Associated with a Decrease in Intraprostatic Concentrations of Androgens **(Table 2)**

In our experiments, dogs treated with 6μg/kg/day selenomethionine had a significant reduction in intraprostatic concentrations of testosterone. The possibility that selenium may exert beneficial effects on the prostate by suppressing androgens deserves further study.

EFFECT OF DHEA ON MOLECULAR BIOMARKERS OF CARCINOGENESIS WITHIN THE AGED DOG PROSTATE

A. DHEA Supplementation Exerts a DNA Damage-Sparing Effect within the Prostate Despite Increased Circulating Serum Dihydrotestosterone (DHT) Concentrations **(Table 3)**

Our original motivation for studying DHEA in the dog model was to determine if DHEA supplementation might exert beneficial or detrimental effects on cellular processes within the aged prostate. DHEA could serve as a precursor for testosterone and DHT. Thus, supplementation with DHEA could aggravate pro-carcinogenic events within the aged human prostate. Using comet assay, we determined the percentage of severely DNA damaged prostate epithelial cells from prostate tissue collected from dogs that received 6 months of treatment with DHEA or no treatment. DHEA supplementation (100mg/kg) of elderly sexually intact male beagle dogs for 6 months resulted in a significant 33% reduction in DNA damage within the prostate compared to sexually intact age-matched controls ($p=0.001$). DHEA treated dogs had a significant 10X increase in mean serum DHT compared to age-matched control dogs ($p=0.02$) Our data suggest that there is a "disconnect" between the concentration of circulating androgens and the accumulation of intraprostatic DNA damage in DHEA treated dogs.

Task 2: Submit proposal for Phase II funding to study the effect of selenium supplementation on molecular biomarkers of carcinogenesis in the aged dog prostate.

After completion of our 6 month laboratory study in Phase I, we initially intended to conduct a 2 year chemoprevention study in pet dogs that would be supported during a Phase II award. Recruitment of elderly sexually intact male pet dogs for this study was scheduled to take place months 12-18. *We changed the scientific direction of the proposed Phase II studies to develop further the 6 month laboratory intervention model rather than using pet dogs.* Three factors influenced this decision:

(1) The National Cancer Institute's commitment to selenium as a prostate cancer chemopreventive agent: The PCPT-2 SELECT trial will study 32,000 men beginning in the year 2000, yet the mechanisms for selenium's cancer protective effect remain unknown.
(2) Our progress indicated that 6 months experimental treatment of purchased elderly beagle dogs provides a unique model system for determining the extent to which selenium changes cellular and molecular biomarkers that are believed to be important in human prostate carcinogenesis. It would be impossible to achieve the same level of control over potentially confounding factors using owned pet dogs.
(3) Reviewer comments from the peer-review panel report of our Phase I proposal expressed concern that pet dog studies were unlikely to yield meaningful information unless conducted under stringent laboratory conditions. Reviewers also had concern with the use of HGPIN as a surrogate endpoint biomarker because there is currently no irrefutable proof that HGPIN in human or dog is the precursor of prostate cancer.

In March 2001, we submitted a Phase II proposal that we believed would best complement the ongoing human trials and assist in establishing the mechanisms of selenium's prostate cancer protective effect. Our Phase II proposal "Effect of Selenium Supplementation on Molecular Biomarkers of Carcinogenesis in the Aged Dog Prostate" was successfully funded by the USAMRMC and is now underway.

Task 3. Complete data analysis from this Phase I study and prepare to conduct the funded Phase II project

We anticipate that data analysis will be completed within the next 4 months. We are preparing 3 manuscripts for publication and anticipate submitting them for publication within the next six months. Requests for lab animal facilities and Animal Care and Use documents for our Phase II project have been completed and approved.

KEY RESEARCH ACCOMPLISHMENTS

- Selenium Supplementation Results in a Significant Reduction in Accumulation of DNA Damage Within the Aged Dog Prostate (Table 1)

- DNA Damage-Sparing Effect of Selenomethionine in the Aged Prostate Occurs Without Upregulation of Serum Activity of the Antioxidant Enzyme Glutathione Peroxidase (Table 1)

- Selenium Supplementation May Decrease DNA Damage Within the Prostate by Upregulation of Apoptosis

- Selenomethionine Supplementation is Associated with a Decrease in Intraprostatic Concentrations of Androgens (Table 2)

- DHEA Supplementation Exerts a DNA Damage-Sparing Effect within the Prostate Despite Increased Circulating Serum Dihydrotestosterone (DHT) Concentrations (Table 3)

- DHEA Supplementation Significantly Decreases DNA damage in Brain and Peripheral Blood Lymphocytes of Elderly Dogs (Table 4)
  These data suggest that DHEA is a neuroactive steroid with anticancer activity that may have potential beneficial effects on the brain.

- Surgical Castration Decreases the Accumulation of DNA Damage in the Aged Brain (Table 5)
  This provides the first evidence that the testis may regulate the steady state level of DNA damage in the aged mammalian brain and may have important implications for brain aging.

REPORTABLE OUTCOMES

Manuscripts

Shen S Cooley DM, Glickman LT, Glickman N, Waters DJ. Reduction in DNA damage in brain and peripheral blood lymphocytes in elderly dogs after treatment with dehydroepiandrosterone (DHEA). *Mutat Res* (in press 2001).

Waters DJ, Shen S, Glickman LT. Life expectancy, antagonistic pleiotropy, and the testis of dogs and men. *Prostate* 2000;43:272-277.

DNA damage-sparing effect of selenium supplementation on peripheral blood lymphocytes in elderly male dogs was published in the Proceedings of the 1999 Cornell Nutrition Conference, p. 55-57.

Patents

Two provisional patents have been filed:

1. Mitigation of DNA Damage in Mammals. Waters DJ, Glickman LT, and Shen S.
2. Mitigation of DNA Damage by Interventions that Antagonize the Testis. Waters DJ, Glickman LT, Shen S.

Poster and Oral Presentations

Extent of DNA Damage in Prostate of Elderly Dogs is Abrogated by Selenomethionine Supplementation. Environmental Mutagen Society Annual Meeting, San Diego, CA, March 2001.

Reduction in DNA damage in brain and peripheral blood lymphocytes of elderly dogs supplemented with dehydroepiandrosterone (DHEA). International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis, Grand Rapids, MI, September 2000.

The Testis Contributes to the Accumulation of DNA Damage in the Aging Mammalian Brain. 29th Annual Meeting of American Aging Association, Boston, MA, June 2000.

Reduction in DNA Damage in Brain and Peripheral Blood Lymphocytes of Elderly Dogs Supplemented with DHEA. The Gerontological Society of America 52nd Annual Scientific Meeting, San Francisco, CA, November 1999.

Reduction in DNA Damage in Peripheral Blood Lymphocytes of Elderly Male Dogs Receiving DHEA Supplementation or Orchiectomy. Serono Symposium on Endocrinology of Aging, Tempe, AZ, October 1999.

Dose-dependent Reduction in DNA Damage in Peripheral Blood Lymphocytes of Dogs by Dietary Selenium Supplementation. American Institute for Cancer Research 9th Annual Research Conference, Washington, DC, September 1999.

Career Development/Research Opportunities

Based on research supported by this award, the PI, Dr. Waters, was awarded 1 of 4 prestigious Brookdale National Fellowship Awards in 1999. The Brookdale Fellowship recognizes individuals with high potential for leadership in Gerontology and supports Dr. Waters' research focus on aging and prostate cancer. As a result of the Phase I Department of Defense award and Brookdale Fellowship, Dr. Waters has been released from clinical responsibilities to concentrate his effort on defining the utility of the dog model of prostate cancer as a model to study prostate aging, and the in vivo testing of potential prostate cancer chemopreventive agents.

CONCLUSIONS

During our Phase I funding period, we have generated data that begins to define the possible mechanism of prostate cancer protective effects of 2 chemopreventive agents, DHEA and selenomethionine. Our results challenge existing paradigms regarding the mechanisms of selenium supplementation and the "dangers" of DHEA supplementation. Our progress to date demonstrates the utility of the dog model in prostate cancer prevention research. We have also made important observations on the effects of these cancer chemopreventive agents on essential organs, such as brain. During the next 12 years, the National Cancer Institute sponsored PCPT-2 SELECT trial will study more than 32,000 men to evaluate whether selenium +/- vitamin E will decrease the incidence of human prostate cancer. Despite a growing body of evidence to support selenium as a potential prostate cancer chemopreventive agent, the molecular mechanism by which selenium might modulate key events in the multistep process of prostate carcinogenesis remains unclear. The goal of our Phase II proposal is to further define the possible mechanism by which selenium supplementation exerts a prostate cancer protective effect. Our work to date, together with our proposed Phase II project, takes an important step toward generating important and useful information necessary to develop selenium as a practical means of prostate cancer chemoprevention. Our research addresses a key underexplored area – the further development of an animal model system to study the effects of potential chemopreventive agents on cellular processes within the prostate that may be critical to human prostate carcinogenesis. Importantly, this work will complements ongoing human chemoprevention trials and may guide the design of future trials in terms of selenium form, dose, interactions with other chemopreventive agents, and in the selection of selenium-responsive biomarkers within the prostate.

REFERENCES

None

APPENDIX

Tables:

Manuscripts

Shen S Cooley DM, Glickman LT, Glickman N, Waters DJ. Reduction in DNA damage in brain and peripheral blood lymphocytes in elderly dogs after treatment with dehydroepiandrosterone (DHEA). *Mutat Res* (in press 2001).

Waters DJ, Shen S, Glickman LT. Life expectancy, antagonistic pleiotropy, and the testis of dogs and men. *Prostate* 2000;43:272-277.

DNA damage-sparing effect of selenium supplementation on peripheral blood lymphocytes in elderly male dogs was published in the Proceedings of the 1999 Cornell Nutrition Conference, p. 55-57.

Poster Abstracts

Extent of DNA Damage in Prostate of Elderly Dogs is Abrogated by Selenomethionine Supplementation. Environmental Mutagen Society Annual Meeting, San Diego, CA, March 2001.

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Reduction in DNA Damage in Peripheral Blood Lymphocytes of Elderly Male Dogs Receiving DHEA Supplementation or Orchiectomy. Serono Symposium on Endocrinology of Aging, Tempe, AZ, October 1999.

Dose-dependent Reduction in DNA Damage in Peripheral Blood Lymphocytes of Dogs by Dietary Selenium Supplementation. American Institute for Cancer Research 9th Annual Research Conference, Washington, DC, September 1999.

Table 1. DNA Damage Sparing Effect of Selenomethionine in the Aged Prostate Occurs Without Upregulation of Serum Activity of the Antioxidant Enzyme Glutathione Peroxidase (GPX)

Group	Mean (± SD) % of cells within prostate with extensive DNA damage	Mean (± SD) serum GPX activity (nm/min/mg)
Intact Control (n=8 dogs)	80 ± 5	24.55 ± 6.56
Selenomethionine*		
3μg/kg/day (n=10 dogs)	58 ± 9[a]	21.74 ± 4.64
6μg/kg/day (n=9 dogs)	62 ± 14[a]	24.21 ± 4.13

*The 3μg/kg/day and 6 μg/kg/day doses of selenomethionine correspond to 200μg and 400μg daily human dose, respectively.

[a]Statistically different from intact control dogs at $p<0.0001$.

Table 2. Selenium Supplementation is Associated with a Reduction in Intraprostatic Concentration of Testosterone

Group	Serum Testosterone (ng/ml)		Prostate Testosterone (pg/g)
	Pre-treatment	6 months	6 months
Intact Control (n=5 dogs)	1.85 ± 0.91	1.59 ± 0.63	1087 ± 584
Selenomethionine*			
3μg/kg/day (n=5 dogs)	2.17 ± 0.93	2.16 ± 1.11	1039 ± 385
6μg/kg/day (n=5 dogs)	2.00 ± 0.938	0.84 ± 0.28**	345 ± 151[a]

*The 3μg/kg/day and 6 μg/kg/day doses of selenomethionine correspond to 200μg and 400μg daily human dose, respectively.

**42% reduction from pre-treatment serum testosterone level ($p=0.06$).

[a]Significantly different from intact control at $p=0.03$.

Table 3. DHEA Supplementation Exerts a DNA Damage-Sparing Effect within the Aged Prostate Despite Increased Serum Dihydrotestosterone (DHT)

	Mean (± SD) % of prostate cells with extensive DNA damage	Serum DHT (pg/ml)
Intact Controls (n=8 dogs)	80 ± 5 %	1289 ± 730
DHEA Supplementation (n=8 dogs)	62 ± 12 %[a]	147 ± 99 [b]

[a] Differs significantly from intact controls at p=0.0017
[b] Differs significantly from intact controls at p=0.02

Table 4. Reduction in Basal Level of DNA Damage in Brain Tissue and Peripheral Blood Lymphocytes (PBLs) of Elderly Dogs Receiving DHEA Supplementation

	MEAN (± SD) % OF CELLS WITH EXTENSIVE DNA DAMAGE	
	Brain Tissue	PBLs
Control Group (n=9 dogs)	78 ±7%	20 ± 2%
DHEA Supplementation (n=8 dogs)	45 ± 10%[a]	10 ± 2% [a]

[a] Differs from control group at p<0.0001.

Table 5. Orchiectomy Reduces Basal Level of DNA Damage in Brain Tissue and Peripheral Blood Lymphocytes of Elderly Male Dogs

	Mean (± SD) % of cells with extensive DNA damage	
BRAIN		
Sexually intact male dogs (n=9)	78 ± 7%	p<0.0001
Orchiectomized dogs (n=9)	37 ± 11%	
PERIPHERAL BLOOD LYMPHOCYTES		
Sexually intact male dogs (n=9)	20 ± 2%	p<0.0001
Orchiectomized dogs (n=9)	11 ± 2%	

Reduction in DNA Damage in Brain and Peripheral Blood Lymphocytes of Elderly Dogs After Treatment with Dehydroepiandrosterone (DHEA)

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Running Title: DHEA and DNA Damage

KEY WORDS: aging, brain, DNA damage, single cell gel electrophoresis, lymphocytes

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ABSTRACT

DNA damage may contribute to organismal senescence and an increased risk for specific age-related diseases. The adrenal steroid, dehydroepiandrosterone (DHEA), exhibits significant anticancer activity in rodent models and has neuroactive and antioxidant properties. However, the influence of DHEA supplementation on tissue-specific DNA damage has not been previously investigated. The purpose of this study was to use alkaline comet assay to determine if DHEA treatment significantly reduces the extent of DNA damage in brain or peripheral blood lymphocytes (PBLs). Elderly male dogs were randomly assigned to receive no treatment (sexually intact controls; n=9 dogs) or DHEA (100mg/kg per os daily; n=8 dogs). Mean (± sd) percentage of brain cells or PBLs with extensively damaged DNA was compared between treatment groups. The susceptibility of PBLs to H_2O_2-induced DNA damage was also measured. After 7 months of treatment, 45 ± 10% of cells from the brain of DHEA treated dogs had extensive DNA damage, compared to 78 ± 7% of brain cells of control dogs (p=0.0005). DHEA treated dogs also had a significant reduction in basal DNA damage in PBLs; mean percentage of PBLs with extensive basal DNA damage was 10 ± 2% compared to 20 ± 2% in the control group (p=0.0005). After 7 months of treatment, PBLs of dogs in the DHEA treated group were more than twice as resistant to H_2O_2-induced DNA damage compared to PBLs of control dogs (p=0.0008). Extent of basal DNA damage in PBLs was not a strong predictor of DNA damage within the brain. These findings suggest that DHEA supplementation can significantly reduce steady state levels of DNA damage in the mammalian brain. Further evaluation of DHEA as a neuroactive agent and its effects on DNA damage and gene expression in other tissues and species is warranted.

INTRODUCTION

Steady state levels of DNA damage are substantial in vertebrate animals as a consequence of exposure to endogenous and environmental mutagens [1,2]. It has been hypothesized that DNA damage contributes to organismal senescence and the increased risk for specific age-related diseases [1,3-5]. Post-mitotic cells in somatic tissues, such as the brain, may be particularly susceptible [3,6]. An accumulation of DNA damage has been implicated in the age-related functional decline of the human brain [7] and increased DNA damage has been reported in patients with neurodegenerative diseases, such as Alzheimer's disease [8,9].

Human aging is accompanied by a profound decline in plasma levels of the adrenal steroid, dehydroepiandrosterone (DHEA) and its sulfate, DHEA-S [10]. Beneficial effects of exogenous DHEA in humans have been reported [11] and increasing evidence suggests that DHEA is a neuroactive agent [12] that influences important cellular processes, such as lipid peroxidation within the aging brain [13,14]. However, to our knowledge, the influence of DHEA supplementation on DNA damage within the brain or other tissues has not been reported.

Because DHEA exhibits anticarcinogenic [15] and antioxidant [13] properties in experimental systems, we hypothesized that DHEA treatment would reduce tissue-specific DNA damage in elderly dogs. In this study, we focused on the brain due to growing evidence that DHEA is a neuroactive steroid and because ongoing human trials are evaluating the effect of DHEA treatment on well-being, cognitive performance, and memory. We studied elderly male dogs, physiologically equivalent to 59 to 69-year old men [16], to determine if long term

(7 month) daily treatment with DHEA could modulate cellular processes which might contribute to age-related decline in brain function. Using alkaline comet assay, we pursued three specific aims: (1) to determine if DHEA treatment significantly decreases basal DNA damage within the aged brain; (2) to determine if DHEA treatment reduces basal DNA damage in peripheral blood lymphocytes and enhances their resistance to oxidant stress; and (3) to determine if the extent of DNA damage within peripheral blood lymphocytes of DHEA treated or untreated dogs is correlated with the extent of DNA damage within the brain. The results of this study indicated that treatment of elderly male dogs with DHEA has significant DNA damage sparing effects on the brain and peripheral blood lymphocytes.

MATERIALS AND METHODS

1. Chemical Reagents

RPMI-1640 medium (with L-glutamine and without sodium bicarbonate), disodium EDTA, Hanks' balanced salts (without calcium chloride, magnesium sulfate and sodium bicarbonate), Dulbecco's phosphate buffered saline (PBS, without calcium chloride and magnesium chloride), dimethylsulfoxide (DMSO), triton X-100, trizma base, trypan blue, Histopaque 1119, sodium chloride, sodium hydroxide, hydrogen peroxide, Ficoll (Type 400) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Low and normal melting point agarose were supplied by Fisher (Fair Lawn, NJ). Fetal bovine serum (FBS) was obtained from Intergen (Purchase, NY). SYBR Green I was from Molecular Probes, Inc. (Eugene, OR).

2. Experimental Animals

Seventeen elderly (8.5-10.5 year old) sexually intact male retired breeder dogs weighing 9 to 18 kg were purchased from a local supplier. All dogs were in good health upon initial physical examination. After 4 weeks acclimation, dogs were randomly assigned to one of two groups: no treatment (sexually intact controls; n=9 dogs); or daily oral supplementation with 100 mg/kg DHEA (Genelabs Technologies, Inc., Redwood City, CA) (DHEA group; n=8 dogs). All dogs were fed a maintenance diet (Science Diet® Canine Maintenance, Hills Pet Nutrition, Inc., Topeka, KS) throughout the acclimation period and 7 month experimental period. At the end of the experiment, all dogs were euthanatized in accordance with guidelines set forth by the AVMA Panel on Euthanasia. All aspects of this experimental protocol were approved by the Purdue University Animal Care and Use Committee.

3. Sampling

Brain - Immediately after euthanasia, brain tissue from the cerebral cortex was collected via craniotomy. In all cases, interval from euthanasia to brain tissue harvest was less than 30 minutes. For each dog, 50-80 mg of brain tissue was placed in 1 ml of cold Hanks' balanced salt solution (HBSS) containing 20 mM EDTA and 10% DMSO [17]. The tissue was then minced with fine scissors and 50 µl of cell suspension was mixed with 1 ml of RPMI 1640 media containing 10% FBS for subsequent electrophoresis.

Peripheral blood lymphocytes (PBLs) - Whole blood (5 ml) was obtained by venipuncture from each dog 7 months after treatment. In 9 dogs (5 control, 4 DHEA treated), blood samples were also collected prior to treatment. Freshly harvested PBLs were obtained using a Ficoll/Hypaque technique described previously for the isolation of canine PBLs [18,19]. Briefly, Ficoll/Hypaque was prepared to yield a solution with a specific gravity of 1.066 as confirmed by a calibrated hydrometer. Two ml of Ficoll/Hypaque 1.066 were layered on top of 2 ml of Histopaque (specific gravity 1.119) in 15 ml polypropylene tubes. Five ml of whole blood (EDTA) and 5 ml of phosphate buffered saline (PBS) were mixed and layered onto the Ficoll/Hypaque gradient. The tubes were centrifuged at 400 x *g* for 20 minutes. The mononuclear cell band in the gradient was removed and washed twice in PBS with BSA. Isolated mononuclear cells were then resuspended in RPMI 1640 media supplemented with 10% FBS and incubated in 5% CO_2 at 37°C for 1.5 hours. Non-adherent cells were collected and cytospin preparations confirmed >90% lymphocytes in this enriched cell population. Mean percentage cell viability, estimated by trypan blue dye exclusion, was 91%.

4. Alkaline Comet Assay

DNA damage was detected by single cell gel electrophoresis (comet assay) based on the method of Singh *et al.* [20]. To determine the extent of basal DNA damage, brain cells or PBLs were suspended in low melting point agarose in PBS at 37°C and pipetted onto a glass microscope slide pre-coated with a layer of normal melting point agarose. The final layer was comprised of 80 µl of low melting agarose alone. After solidification of the agarose, slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris and 300 mM NaOH to adjust the pH to 10.0, 10% DMSO and 1% Triton X-100 added fresh) and stored in the dark overnight at 4 °C. Slides were then removed from the lysing solution and placed on a horizontal gel electrophoresis tank (Fisher, Fair Lawn, NJ) containing freshly prepared alkaline buffer (300 mM NaOH and 1 mM Na_2 EDTA, pH > 13). Slides remained submerged in buffer for 20 minutes before electrophoresis at 25 V and 300 mA for 30 minutes. Slides were then washed three times (five minutes each) with 0.4M Tris at pH 7.5. After the final wash, slides were drained and exposed to cold 100% ethanol to dry. All steps from cell lysis until the end of neutralization were performed in the dark or under yellow light. Each slide was stained with 150 µl of SYBR Green 1 (1:10,000 dilution in TE buffer at pH 7.5) prior to analysis. To determine if treatment of dogs with DHEA affected the sensitivity of their PBLs to oxidant stress, freshly isolated PBLs from each dog were exposed to 25µM H_2O_2 for 5 minutes at 4°C prior to suspension in agarose and electrophoresis.

5. Image Analysis

Under the conditions of this experiment, the comet tail results from migration of DNA fragments that represent strand breaks, alkali labile sites, crosslinks, and base excision repair sites [20]. Extent of DNA damage was scored in 100 randomly selected cells from each dog (50 cells per duplicate slide) by an examiner who was blinded to treatment group. SYBR Green I-stained nucleoids were examined at 200X magnification with an Olympus epifluorescent microscope. Each cell was visually scored on a 0 to 4 scale according to its appearance using a method described by Collins [21,22]. Each cell was scored as follows: no damage (type 0); mild to moderate damage (type 1 & 2); and extensive DNA damage (type 3 & 4) **(Figure 1a)**. Using this scoring method, the extent of DNA damage within brain or PBLs was expressed in terms of a comet score (range 0-400) [21] and as the percentage of extensively damaged cells (sum of type 3&4 cells).

6. Statistical Analysis

All data analyses used standard statistical software (SAS System Version 8.1. SAS Institute, Inc., Cary, NC, 1999) and differences were considered to be statistically significant at $p < 0.05$. Mean basal DNA damage within the brain of control dogs and DHEA treated dogs was expressed in terms of comet score and percentage of extensively damaged cells. Similarly, extent of DNA damage in PBLs with or without *in vitro* H_2O_2 challenge prior to electrophoresis was expressed in terms of comet score and percentage extensively damaged cells. In addition, we used the percentage extensively damaged cells to calculate an H_2O_2-inducible DNA damage index ($IDDI_{H2O2}$) as follows: $IDDI_{H2O2}$ = (H_2O_2 damage – basal damage) / (100 – basal damage). This index allowed us to detect differences in the amount of H_2O_2-inducible DNA damage

measured for each dog that might otherwise be obscured by intersubject differences in basal DNA damage. Kruskal Wallis tests were performed to determine if the extent of DNA damage in brain (basal) or in PBLs (basal, H_2O_2-induced, $IDDI_{H2O2}$) collected at 7 months was significantly different between treatment groups (PROC TTEST, SAS/STAT User's Guide, Version 8, Volume 2. SAS Institute, Inc., Cary, NC, 1999, pp. 3567-3590). In 9 dogs (5 controls, 4 DHEA treated), repeated measures analysis of variance (PROC GLM, SAS/STAT User's Guide, Version 8, Volume 2. SAS Institute, Inc., Cary, NC, 1999, pp. 1465-1636) were performed to determine if there were significant differences in the extent of DNA damage over the treatment period using PBLs collected at baseline and 7 months post-treatment. In order to determine if the extent of DNA damage in PBLs could reliably predict the extent of DNA damage within the brain, Pearson correlation coefficients were calculated separately for each treatment group for basal DNA damage in brain and PBLs collected at 7 months (PROC CORR, SAS Procedures Guide, Version 8, Volume 1. SAS Institute, Inc., Cary, NC, 1999, pp. 273-312).

RESULTS

Effect of DHEA Treatment on Extent of DNA Damage in Brain – Dogs treated with DHEA for 7 months had a significant reduction in the extent of basal DNA damage within the brain **(Figure 1b-d and Table 1).** DHEA treated dogs had a 42% reduction in the mean percentage of cells with extensively damaged DNA (p=0.0005) and a 25% reduction in mean comet score (p=0.0005).

Effect of DHEA Treatment on Extent of DNA Damage in Peripheral Blood Lymphocytes (PBLs) - Dogs supplemented with DHEA had a significant reduction in the extent of basal DNA damage within PBLs **(Table 1).** DHEA treated dogs had a 50% reduction in the mean percentage of cells with extensively damaged DNA (p=0.0005). After 7 months of treatment, the PBLs of DHEA treated dogs were significantly less vulnerable to H_2O_2-induced DNA damage. DHEA supplementation was associated with a two-fold reduction in the percentage of PBLs with extensively damaged DNA after H_2O_2 exposure (p=0.0008), and a greater than two-fold reduction in the H_2O_2-inducible DNA damage index (p=0.0005). Prior to treatment, there were no significant differences between treatment groups in basal or H_2O_2-induced DNA damage. However, a significant time-group effect was seen for each of the DNA damage parameters in PBLs, indicating a significant reduction in DNA damage over the experimental period in DHEA treated dogs compared to control dogs. The significant changes in DNA damage in DHEA treated dogs measured in PBLs collected at baseline and after 7 months treatment are summarized in **Table 2**.

Correlation Between the Extent of Basal DNA Damage in Brain and Peripheral Blood Lymphocytes – In control dogs, the extent of basal DNA damage (comet score) in PBLs was not significantly correlated with the extent of basal DNA damage in the brain ($r = -0.27$; $p=0.47$). In DHEA treated dogs, there was a negative correlation between basal level of DNA damage in PBL and brain ($r = -0.51$; $p=0.19$). Therefore, the extent of DNA damage within a subject's PBLs was a weak predictor of DNA damage within the brain (**Figure 2**).

DISCUSSION

The purpose of this study was to determine if DHEA supplementation could reduce tissue-specific DNA damage in elderly dogs. Using the alkaline comet assay, we found that elderly male dogs receiving daily DHEA treatment for 7 months had significantly reduced basal levels of DNA damage detectable in their brain compared to age-matched control dogs. To our knowledge, this is the first study to demonstrate that DHEA can significantly reduce DNA damage in the mammalian brain. DHEA treatment also significantly reduced the extent of basal DNA damage in PBLs and increased the resistance of PBLs to oxidant stress.

DHEA is a pleiotropic steroid that is widely available in the United States as an over-the-counter supplement. The well documented age-related decline in plasma DHEA and DHEA-S concentrations in humans [10,23,24] has generated considerable speculation that DHEA supplementation of elderly adults might slow or even reverse some of the undesirable physiologic changes associated with aging. The possibility that DHEA might exhibit health promoting effects is supported by experimental evidence in animals that DHEA exerts cancer preventive activity [25-28] and restores age-related dysregulation of immunoregulatory cytokines [29-30]. Increasing experimental evidence suggesting that DHEA has neuroactive properties [12,31] prompted us to study the effect of DHEA treatment on the aged dog brain, because it represents a potentially useful naturally-occurring mammalian model to study early neuronal changes associated with aging, the initial stages of senile plaque formation, and age-related cognitive dysfunction [32-38]. The elderly dogs in our study were physiologically equivalent to 59 to 69-year old men. Under carefully controlled conditions, we determined if DHEA treatment could alter steady state levels of DNA damage within the brain.

Previous work suggested that DHEA is a neuroactive steroid capable of modulating cellular and molecular processes within the brain. The best documented effects of DHEA on the central nervous system are its ability to act as a GABA receptor antagonist and an agonist of sigma receptors [31,39]. Ex vivo studies have shown that DHEA can protect human brain tissue from $H_2O_2/FeSO_4$-induced lipid peroxidation [14,40]. These results are consistent with data from hyperglycemic rats in which DHEA treatment significantly reduced lipid peroxidation within the brain [13,41]. Our results provide the first evidence that DHEA treatment can reduce the extent of DNA damage within the aged brain. After 7 months of treatment, DHEA treated dogs had an almost two-fold reduction in the percentage of cells from cerebral cortex with extensive DNA damage, levels comparable to the extent of basal DNA damage measured in the brain of young, 1 to 2-year old dogs (data not shown). It is plausible that age-related increases in DNA damage within the brain [42-46] contribute to age-related decline in cognitive function. Increased DNA damage has been reported in the brain [4,8] and PBLs [9] of Alzheimer's disease patients. DNA damage may interfere with transcription and if not repaired might contribute to increased molecular disorder within the aged brain. The resultant disruption of homeostasis might also increase the risk for neurodegenerative disease.

It is not known if interventions that spare DNA damage lead to improved cognitive function in humans or animals. However, several studies in rodents suggest that DHEA treatment exerts potent anxiolytic and memory-enhancing effects; these studies have been reviewed recently [39]. DHEA supplementation in humans was associated with an increased sense of well being in one study [11], and Alzheimer's disease patients with the highest plasma

concentration of DHEA-S performed better on certain memory function tests [47]. In contrast, no significant association was found between plasma DHEA-S level and cognitive function or memory in healthy aged human subjects [48]. To date, clinical trials in humans conducted to evaluate the ability of DHEA treatment to enhance memory or cognitive function have not yielded positive results [49,50]. These negative results may reflect suboptimal dose or duration of treatment, use of insensitive outcome measures, or that the subset of the human population that might benefit most from DHEA supplementation has not yet been defined.

The DNA damage-sparing effect of DHEA treatment on the brain of elderly male dogs was accompanied by a reduction in the extent of DNA damage in PBLs. Dogs treated with DHEA for 7 months had a two-fold decrease in the extent of basal DNA damage in PBLs compared to pre-treatment levels. Similarly, after 7 months of treatment, basal DNA damage in PBLs of DHEA treated dogs was two-fold less than in age-matched control dogs. We also found that DHEA treatment rendered PBLs more resistant to H_2O_2-induced oxidant stress. These findings suggest that the DNA damage-sparing effect of DHEA in PBLs, and perhaps in brain, may be in part mediated by its antioxidant properties. The mechanism by which DHEA exerts this antioxidant effect may involve upregulation of intracellular NADPH levels by inhibition of glucose-6-phosphate dehydrogenase [51].

In this study, we used single cell gel electrophoresis (alkaline comet assay) to measure DNA damage in brain and PBLs. We expressed the extent of basal DNA damage in terms of a comet score, in which 100 cells from each dog were scored on a 0 to 4 scale according to their appearance. This scoring system yields reproducible results in our hands and has been

shown by other investigators [52] to correlate well with other measures of DNA damage assessment obtained using image analysis software (e.g. tail moment). We also expressed DNA damage in terms of percentage of cells with extensive DNA damage (type 3&4 cells). DHEA treatment exhibited significant DNA damage-sparing effects as measured by either of these parameters. In addition to measuring the extent of DNA damage in PBLs after H_2O_2 challenge, we described the response of PBLs to H_2O_2-induced oxidant stress in terms of an inducible DNA damage index. This index compares the response to H_2O_2 challenge in DHEA treated vs. age-matched controls after standardizing for the significant difference in basal DNA damage between the two groups. By this method, only cells without extensive basal DNA damage are considered susceptible to H_2O_2-induced damage. In untreated dogs, H_2O_2-inducible DNA damage index was remarkably stable over the 7 month experimental period (34.6% at 0 months vs. 31% at 7 months). In contrast, after 7 months treatment with DHEA, only 15% of susceptible PBLs underwent H_2O_2-induced extensive DNA damage.

Comet analysis of PBLs has been used extensively to assess DNA damage resulting from environmental, occupational, or lifestyle exposures and to identify potential protective dietary factors that might reduce DNA damage [2,53]. The biological importance of the DNA alterations in PBLs detected by comet assay is undetermined, since it is not known whether the extent of DNA damage in PBLs offers a reliable assessment of DNA damage in vital organs, such as brain or heart, or non-essential organs susceptible to carcinogenesis, such as breast or prostate. To pursue this question, we tested whether basal DNA damage within PBLs was strongly correlated with the extent of basal DNA damage within the brain. In elderly male dogs, we found an unexplained negative correlation ($r = -0.51$) between basal DNA damage in PBLs

and basal DNA damage within the brain of DHEA treated dogs. A weaker negative correlation existed between the extent of basal DNA damage within PBLs and brain of untreated dogs. Further studies are needed to evaluate whether the predictive value of PBLs as surrogate indicator cells is dependent upon the particular genotoxin or protective factor under investigation. Our findings would suggest that the relationship between the extent of basal DNA damage in brain and PBLs in elderly male dogs is altered by treatment with the protective factor, DHEA. At this point, our findings do not support the utility of using PBLs as a non-invasive surrogate to monitor basal DNA damage within the mammalian brain. However, because significant target tissue-specific differences in the sensitivity to DNA damage or DNA repair capacity may exist, PBLs may still provide useful information on the extent of DNA damage in other tissues, such as breast or prostate.

CONCLUSIONS

Using the alkaline comet assay to measure the extent of DNA damage in cells, we found that elderly male dogs receiving daily DHEA treatment for 7 months had significantly reduced basal levels of DNA damage detectable in their brain compared to age-matched control dogs. Further analysis of the effects of DHEA supplementation on DNA damage and gene expression in brain and other tissues is warranted. DHEA supplementation also significantly reduced the extent of basal and H_2O_2-induced DNA damage in peripheral blood lymphocytes. A long-term objective of this research is to utilize elderly dogs to test the effects of dietary interventions and other treatments on cellular processes, such as accumulation of DNA damage, which are believed to contribute to age-related functional decline and the development of age-related diseases, such as cancer.

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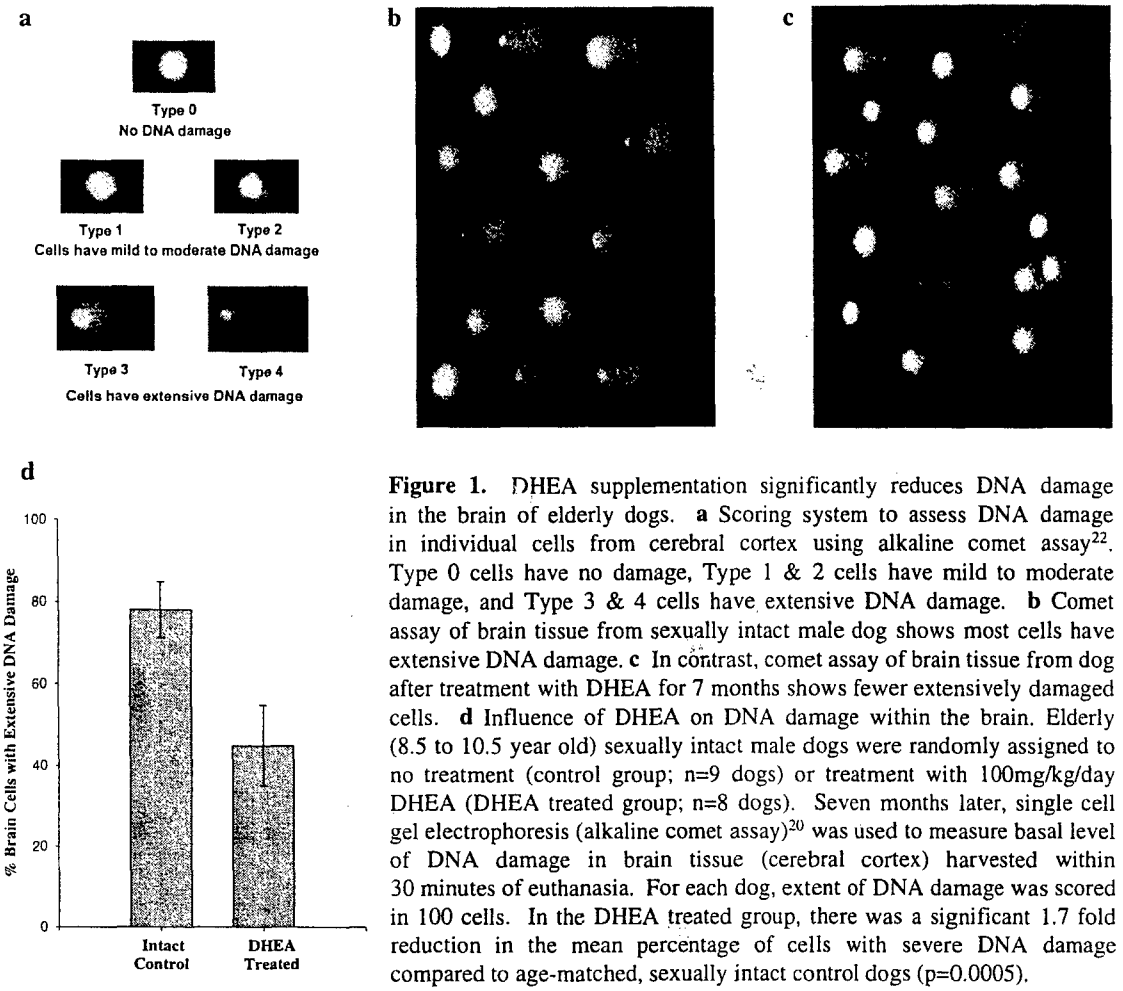
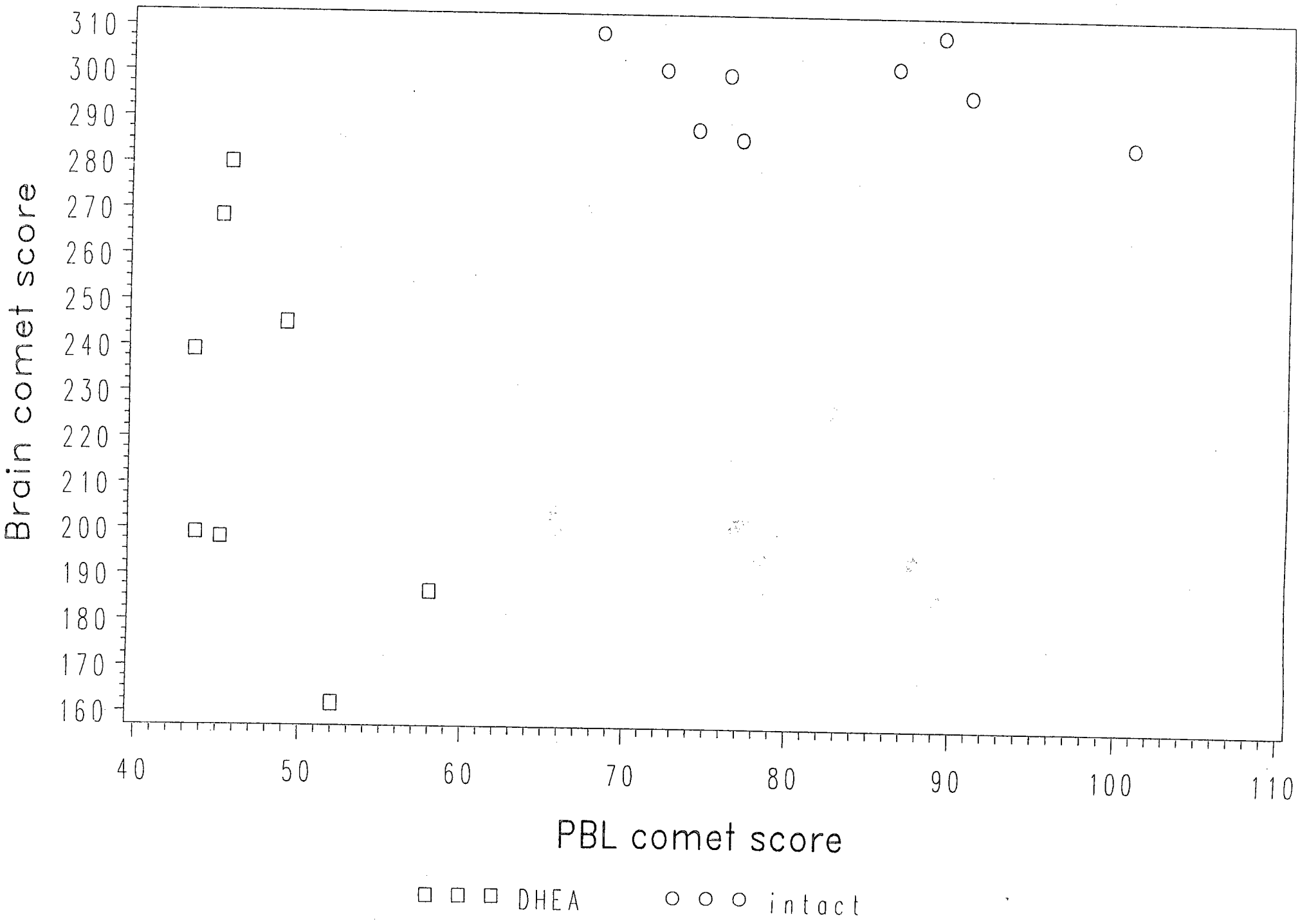


Figure 1. DHEA supplementation significantly reduces DNA damage in the brain of elderly dogs. **a** Scoring system to assess DNA damage in individual cells from cerebral cortex using alkaline comet assay[22]. Type 0 cells have no damage, Type 1 & 2 cells have mild to moderate damage, and Type 3 & 4 cells have extensive DNA damage. **b** Comet assay of brain tissue from sexually intact male dog shows most cells have extensive DNA damage. **c** In contrast, comet assay of brain tissue from dog after treatment with DHEA for 7 months shows fewer extensively damaged cells. **d** Influence of DHEA on DNA damage within the brain. Elderly (8.5 to 10.5 year old) sexually intact male dogs were randomly assigned to no treatment (control group; n=9 dogs) or treatment with 100mg/kg/day DHEA (DHEA treated group; n=8 dogs). Seven months later, single cell gel electrophoresis (alkaline comet assay)[20] was used to measure basal level of DNA damage in brain tissue (cerebral cortex) harvested within 30 minutes of euthanasia. For each dog, extent of DNA damage was scored in 100 cells. In the DHEA treated group, there was a significant 1.7 fold reduction in the mean percentage of cells with severe DNA damage compared to age-matched, sexually intact control dogs (p=0.0005).

LEGEND FOR FIGURE 2

Figure 2. Correlation between extent of DNA damage in brain versus peripheral blood lymphocytes (PBLs) in 8 DHEA treated dogs (□) and 9 untreated control dogs (○). Basal DNA damage in 100 cells was expressed as a comet score (0-400). Pearson correlation coefficients calculated separately for DHEA treated dogs ($r = -0.51$; $p=0.19$) and untreated dogs ($r = -0.27$; $p=0.47$) indicated that the extent of DNA damage in PBLs was not a strong predictor of DNA damage within the brain of elderly male dogs.

Figure 2


Brain comet score
310
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100
110
PBL comet score
DHEA
intact

Table 1. Extent of DNA Damage in Brain Tissue and Peripheral Blood Lymphocytes of Control Dogs and Dogs Receiving 7 Months Treatment with DHEA

	Control Group (n=9 dogs)	DHEA Treated Group (n=8 dogs)	
Brain Tissue			
Basal DNA Damage			
% Extensive Damage*	78 ± 7%	45 ± 10%	p=0.0005
Comet Score**	297.3 ± 9.2	222.2 ± 42.0	p=0.0005
Peripheral Blood Lymphocytes			
Basal DNA Damage			
% Extensive Damage	20 ± 2%	10 ± 2%	p=0.0005
Comet Score	81.8 ± 10.7	47.7 ± 5.1	p=0.0005
H_2O_2-induced DNA Damage			
% Extensive Damage	47 ± 4%	23 ± 8%	p=0.0008
Comet Score	198.7 ± 14.5	128.7 ± 32.3	p=0.0005
Inducible DNA Damage Index***	33.4 ± 4.1%	14.8 ± 7.4%	p=0.0005

*Mean ± sd percentage of cells with extensive DNA damage (Type 3&4)

**Comet score expresses the extent of DNA damage in 100 cells based upon a scale of 0 (lowest) to 400 (highest).

***See text p. 8 for calculation of this index.

Table 2. Effect of DHEA Treatment on Extent of DNA Damage in Peripheral Blood Lymphocytes of Elderly Dogs

	Pre-Treatment	7mo Post-Treatment	
DHEA Supplementation (n=4 dogs)			
Basal DNA Damage			
% Extensive Damage*	21 ± 1%	11 ± 2%	p=0.003****
Comet Score**	91.5 ± 8.0	49.7 ± 6.6	p=0.001
H_2O_2-induced DNA Damage			
% Extensive Damage	58 ± 7%	30 ± 2%	p=0.005
Comet Score	248.0 ± 33.1	157.0 ± 14.1	p=0.007
Inducible DNA Damage Index***	46.6 ± 7.7%	21.6 ± 1.2%	p=0.006

*Mean ± SD percentage of cells with extensive DNA damage (Type 3&4)

**Comet score expresses the extent of DNA damage in 100 cells based upon a scale of 0 (lowest) to 400 (highest).

***See text p. 8 for calculation of this index.

****Significance level from paired analysis of pre-treatment and post-treatment value

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Life Expectancy, Antagonistic Pleiotropy, and the Testis of Dogs and Men

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BACKGROUND. Prostate cancer and benign prostatic hyperplasia are important age-related prostatic diseases that are under the influence of testicular hormones. However, the disparity between male and female life expectancy within the human population cannot be explained solely by the prevalence of prostatic disease-related mortality. The purpose of this paper is to explore the possibility that the testis exerts a detrimental effect on life span.
METHODS. First, we review previously published and unpublished data on the influence of the testis on the life span of dogs and men. Aging in pet dogs and men is then discussed in terms of evolutionary theory, emphasizing the significance of a prolonged postreproductive life span and possible consequences of late-acting deleterious genes in these two species. Finally, we present preliminary data that orchiectomy can reduce DNA damage within the brain of elderly male dogs.
RESULTS AND CONCLUSIONS. Taken together, these observations raise the intriguing possibility that interventions to antagonize the testis might have much broader therapeutic applications that will extend well beyond the treatment of prostate cancer. *Prostate 43:272–277, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **aging; prostate; orchiectomy; brain; DNA damage; single-cell gel electrophoresis; endocrinology; life span; longevity**

THE GIVE AND TAKE OF THE TESTIS: REPRODUCTIVE SUCCESS AT THE EXPENSE OF PROSTATE PATHOLOGY

The testis is unquestionably essential for male reproductive success and the perpetuation of the species. In addition to spermatogenesis, hormones elaborated by the testis play a critical role in virilization of the urogenital tract and the development of male sexual behavior [1,2]. Testicular hormones are also important regulators of homeostasis within the prostate gland. The development, growth, and cytodifferentiation of the prostate are dependent on androgens, and these processes are likely orchestrated by complex, reciprocal stromal-epithelial interactions [3]. Prostatic stimulation by testicular hormones also significantly contributes to age-related prostate pathology. The importance of androgens in the development of benign prostatic hyperplasia (BPH) and in prostate carcinogenesis is widely accepted, although a consistent relationship between serum or tissue androgen levels and risk for these prostatic diseases has not been found [4]. Curiously, BPH and prostatic cancers develop spontaneously in the prostates of aging dogs and men at a life stage when testicular androgen production is declining, suggesting that nonandrogen factors may also be important [5,6]. Experimental manipulations in dogs suggest that androgens and estrogens might act synergistically to induce BPH [7]. Data also support an important role for the testis in determining the vulnerability of the canine prostate to develop BPH in response to endocrine manipulations [8]. It has been proposed that the response of the prostate to sex steroids may be regulated by a yet-to-be-identified testicular or epididymal nonhormonal factor [8–10]. Based on available data, it would appear that simply

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Fig. 1. Comparison of age-specific death rate (ASDR) from birth to 80 years in white males vs. white females. The vertical axis indicates the male survival disadvantage calculated as the gender-specific difference in ASDR expressed in deaths per 1,000 persons alive at the beginning of each year. The disparity between male and female survival increases after 55 years, and even more dramatically within the eighth decade of life. Calculations are from US Census Bureau data [11].

equating the deleterious effect of the testis to the production of androgens is an indefensible oversimplification.

THE STRIKING DISPARITY IN LIFE EXPECTANCY BETWEEN MEN AND WOMEN CANNOT BE SOLELY ATTRIBUTED TO PROSTATIC DISEASE

When the life expectancies of men and women are compared, a consistent male survival disadvantage is apparent. While average life expectancy for men and women has increased, the disparity in life expectancy between the sexes has remained stable over the last 20 years [11]. A closer look at gender-specific survival within the US population reveals that the expected death rate for males exceeds that for females throughout life. Using US Census Bureau data [11], it is possible to compare age-specific death rates of males vs. females, expressed in deaths per 1,000 persons alive at a specified age. Figure 1 shows that the magnitude of the disparity between males and females in age-specific expected death rate dramatically increases after age 55 years, indicating an apparent exacerbation of the male survival disadvantage after middle age.

Could testis-driven prostatic disease be responsible for the male survival disadvantage within the US population? Among aging males, the prevalence of BPH and prostate carcinoma is staggering. In the US, the estimated likelihood that a 50-year-old man will undergo therapeutic intervention for BPH during his lifetime approaches 40% [12]. Prostate cancer accounts for over 25% of cancer incidence and more than 10% of cancer-related mortality in men. Although it is estimated that the life span of the average man who succumbs to metastatic prostate cancer is cut short by 9 years [13], the accelerating male survival disadvantage in aging men cannot be solely attributed to prostatic disease-related mortality. BPH is an almost invariably nonlethal syndrome, and the age-specific prostate cancer mortality rate between ages 70–85 years (the age group in which the incidence of prostate cancer is highest) is approximately 0.2 deaths per 1,000 persons alive [14]. When 0.2 is compared to the gender difference in mortality rate between 70-year-old men and women (13 deaths per 1,000) (Fig. 1), prostatic disease-related mortality accounts for less than 2% of the male survival disadvantage of white US men during the eighth decade of life.

THE INFLUENCE OF THE TESTIS ON HUMAN LIFE SPAN: THE SINGING CASTRATI AND INSTITUTIONALIZED KANSANS

In 1994, Nieschlag et al. [15] published a study that explored the possibility that the testis might contribute to the shortened life span of men. These investigators analyzed life span data from a group of 50 Italian singers who underwent prepubertal surgical castration (orchiectomy) and from a comparison group of sexually intact male singers of the same time period. Mean ± SD life span of the castrates was 65.5 ± 13.8 years compared to 64.3 ± 14.1 years for the intact group. The 1.2-year mean survival advantage of the castrates was not statistically significant. This study was subsequently criticized for its inadequate sample size [16]. Using a sample size of only 50 men in each group, the smallest difference in mean life span that could have been detected with 80% confidence and a probability of type I error of 0.05 would have been 9.1 years. Furthermore, the validity of using a population of Italian men who lived during the period of 1581–1858 was also questioned [17], because the calculated life expectancy of Italian men and women in 1891 was essentially equal (expected age at death of Italian men and women who reached the age of 30 was 64.7 years and 65.0 years, respectively). Unfortunately, the data from these singing castrati fail to implicate or exclude an important role for the testis in determining human life span.

Hamilton and Mestler [18] studied the effect of orchiectomy upon life span among men institutionalized in Kansas for mental retardation. These investigators generated life table data for 735 sexually intact men and 297 men who were castrated between 1895–1950. Age-

specific mortality rates for castrates were significantly lower ($P < 0.01$) than for intact males beginning at age 25 and continuing throughout life. Among white males who were alive at age 40 years, the estimated median life span of men castrated between ages 15–39 years was significantly higher than for intact men (70.7 years for castrates vs. 64.7 years for intact men; $P < 0.001$). The life span-extending effect of castration was most profound in the men who underwent earliest castration; median life span extension was 11.6 years in this subset of castrates compared to intact men. Death certificates indicated that castrated males were less likely to succumb to infectious diseases than intact males. Within this institutionalized population, eunuchs also significantly outlived intact females. The authors suggested that the observed life span-extending effects of orchiectomy were likely to be applicable to the general population, although conceding that "the loss of highly valued organs and function such as the testis and some reproductive behaviors has great psychological impact upon intelligent men" [18].

THE PROLONGED POSTREPRODUCTIVE LIFE SPAN OF DOGS AND MEN: APPLYING EVOLUTIONARY THEORY OF AGING TO DOMESTICATED POPULATIONS

Because of domestication, pet dogs and humans share a prolonged life span, which includes a prolonged postreproductive period of senescence. It is likely that during this postreproductive period, a progressively increasing "molecular disorder" [19] drives the emergence of age-related diseases, such as cancer and neurodegenerative diseases. This hypothesis would correctly predict the low risk for cancer development in feral animals, such as wolves, because their life histories are not characterized by an extended postreproductive senescent period. The disposable soma theory of aging [20] predicts that there would be no advantage for members of a highly domesticated species to invest substantial resources in an attempt to prevent or reverse the molecular disorder that accumulates during the postreproductive senescent period.

In 1957, George Williams proposed the theory of antagonistic pleiotropy, which predicts that certain genes acting in a beneficial manner early in life may exert detrimental effects later in life [21]. Importantly, these pleiotropic genes would never be selectively eliminated from the population and could therefore contribute to age-related functional decline or the development of age-related diseases. Testicular hormones are essential for normal development and male reproduction. Is it possible that the actions of testicular hormones satisfy the criteria of Williams [21] for antagonistic pleiotropy, i.e., the testis exerts detrimental effects on male life expectancy?

As illustrated by the aforementioned studies, testing this hypothesis within the human population would certainly be difficult. However, the importance of studying a domesticated (rather than nondomesticated) population to successfully evaluate the impact of a late-acting deleterious gene was alluded to almost 50 years ago in the infamous "test tube experiment" of Sir Peter Medawar [22]. In an attempt to explain the consequences of age-related deterioration and the action of late-acting deleterious genes on a population, Medawar described an imaginary experiment in which a late-acting deleterious gene induces late-onset disintegration of test tubes. He wrote, "If disintegration (of test tubes) should occur five years after birth, its consequences would be virtually negligible, for ... less than one in 500 of the population (of test tubes) is lucky enough to live so long. Indeed, if we relied upon evidence derived solely from the natural population of test tubes, we should probably never be quite certain that it (a survival disadvantage for test tubes with the late-acting deleterious gene) really happened. We could make quite certain ... only by domesticating our test tubes, shielding them from the hazards of everyday usage by keeping them in a padded box as pets" [22]. Pet dogs represent a domesticated population with an extended postreproductive life span. Because a large percentage of pet dogs undergo elective orchiectomy early in life, pet dogs might serve as a unique model to investigate the influence of the testis on life expectancy.

THE INFLUENCE OF THE TESTIS ON LONGEVITY OF PET DOGS

In order to determine if the testis influences longevity in pet dogs, data were analyzed from a 1997 national health survey of 211 Irish setter dogs (L.T. Glickman, unpublished results). When mean ± SD age at death of sexually intact male and female dogs was compared, a male survival disadvantage was observed. Mean age at death in intact males and females was 9.8 ± 3.1 years and 10.9 ± 2.1 years, respectively ($P = 0.07$). Interestingly, when the mean age at death for Irish setters was expressed as physiologic age in human year equivalents [23], the male survival disadvantage was 5 years for dogs (61 human years for males, 66 human years for females). This closely mimics the male survival disadvantage in the human population, where mean life expectancy at birth for men (74 years) and women (79 years) in 1997 also differs by 5 ± 2.5 years [11]. Mean ± SD life span of orchiectomized male dogs (10.6 ± 2.5 years) exceeded that of intact males. When orchiectomized dogs were

subdivided into tertiles on the basis of their age at orchiectomy, mean age at death was highest for castrated males in the oldest tertile (12.3 ± 1.4 years) compared to intact males ($P = 0.03$). These data support the hypothesis that the testis exerts a detrimental effect on life expectancy. Testis removal, even later in life, might favor an extended life span.

Although intriguing, these data should be interpreted with some caution. Differences in life span between castrates and intact dogs may reflect some as yet unforeseen selection bias. For example, the circumstances that motivate pet owners to castrate their dogs might also be related to longevity. Furthermore, these data from Irish setters do not preclude the possibility that the apparent association between castration and increased longevity in pet dogs might be breed-specific, rather than a universal phenomenon.

TESTING THE HYPOTHESIS THAT ESSENTIAL ORGANS ARE ADVERSELY AFFECTED BY THE TESTIS DURING THE POSTREPRODUCTIVE SENESCENT PERIOD

In order to further examine the relationship between the testis and longevity, we conducted an experiment to determine the effect of orchiectomy on elderly dogs. In this manner, we sought to test whether the theory of antagonistic pleiotropy was operational for testicular hormones. We hypothesized that the testis might contribute to a male survival disadvantage, because testicular hormones exert an adverse effect on an essential organ, thereby significantly affecting life expectancy. To test this hypothesis, we determined if surgical removal of the testes in elderly beagle dogs could reduce the level of DNA damage within the brain. Accumulation of DNA damage has been implicated in age-related functional decline in the human brain [24,25]. DNA damage may also contribute to an increased risk for specific age-related brain diseases, such as Alzheimer's disease. Recently, increased DNA damage was reported in the brain [26] and peripheral blood lymphocytes [27] of patients with Alzheimer's disease compared to age-matched controls.

THE EFFECT OF ORCHIECTOMY ON DNA DAMAGE IN THE BRAIN AND PERIPHERAL BLOOD LYMPHOCYTES OF ELDERLY MALE DOGS

Eight sexually intact male beagle dogs, age 9–10.5 years (62–69 years in human age equivalents [23]), were randomly assigned to two groups: no treatment (control group; n = 4 dogs) or surgical castration (orchiectomized group; n = 4 dogs). Six months later, single-cell gel electrophoresis (comet assay) was used to measure basal levels of DNA damage in brain and peripheral blood lymphocytes. The alkaline comet assay is a robust and versatile assay that has been used to measure DNA damage in a wide variety of normal and transformed cells [28]. The comet assay was performed using a procedure modified from the method of Singh et al. [29]. All experiments used freshly harvested brain tissue (cerebral cortex) or peripheral blood lymphocytes, 20-min alkaline unwinding, and 30 min of electrophoresis (25 V, 300 mA) at pH > 13. Under these conditions, the assay detects single-strand breaks, alkali-labile sites, and sites of base excision repair. Mean time interval from euthanasia to brain tissue harvest was 20 min. For each dog, extent of DNA damage was scored per 100 cells using a modification of the method of Duthie and Collins [30]. Each cell was scored as follows: type 0, no damage; types 1 and 2, mild to moderate damage; and types 3 and 4, extensive DNA damage. Mean (± SD) percentages of extensively damaged cells (types 3 and 4) were compared between treatment groups, using ANOVA (SAS Institute, Cary, NC).

Analysis of brain tissue from orchiectomized dogs 6 months after castration showed that 37 ± 18% of cells had extensive DNA damage (Table I). This was significantly less than the DNA damage in brain tissue of age-matched sexually intact control dogs (82 ± 8% of cells had extensively damaged DNA) ($P = 0.0005$). The orchiectomized group also had a twofold reduction in the percentage of peripheral blood lymphocytes with extensive DNA damage (mean ± SD = 12 ± 2% compared to 24 ± 2% in the control group; $P < 0.0001$).

These preliminary data provide the first evidence that the testis may promote the accumulation of DNA damage within the brain of elderly dogs. These findings are consistent with the hypothesis that testicular hormones contribute to the increasing molecular disorder that is associated with mammalian brain aging. The mechanism by which the testis favors the accumulation of DNA damage within the brain is unknown. It has been shown that physiologic concentrations of androgens increase oxidant stress in human prostate carcinoma cells in vitro [31]. This raises the intriguing possibility that androgens exert prooxidant effects in vivo which may contribute to the accumulation of DNA damage, organismal aging, risk for age-related diseases, and premature death. Our data support the theory that the testis, which is essential early in life, exerts an adverse, antagonistically pleiotropic effect on the host during the postreproductive senescent period. In light of these findings, it is interesting to speculate on the adaptive significance of the age-related decline in circulating testosterone levels of men [32]. Did an aging-related decline in circulating

TABLE I. Orchiectomy Reduces Basal Level of DNA Damage in Brain Tissue and Peripheral Blood Lymphocytes of Elderly Male Dogs*

	Mean (±SD) % of cells with extensive DNA damage	
Brain		
Sexually intact male dogs (n = 4)	82 ± 8%	
Orchiectomized dogs (n = 4)	37 ± 18%	$p = 0.0005$
Peripheral blood lymphocytes		
Sexually intact male dogs (n = 4)	24 ± 2%	
Orchiectomized dogs (n = 4)	12 ± 2%	$p < 0.0001$

*DNA damage assessed 6 months after elderly male dogs were randomized to sexually intact control group vs. orchiectomy group.

testicular hormones "evolve" as a mechanism to protect the brain?

DESIGNING INTELLIGENT INTERVENTIONS TO EVADE A TESTICULAR TRAGEDY

Webster [33] defines tragedy as "a serious play typically dealing with the problems of a central character leading to an unhappy or disastrous ending brought on . . . by fate and a tragic flaw." As average life expectancy is extended in domesticated populations, are we witnessing the evolution of a testicular tragedy? Is the inevitable fate of the sexually intact male population an abbreviated life span mandated by the testis? That testicular androgens might have undesirable effects late in life by stimulating prostatic hyperplasia or by driving the emergence of potentially lethal prostate cancers is not a new concept. However, our data suggest a reconsideration of the potentially adverse effects of the testis on middle-aged and elderly men. It is conceivable that in the postreproductive stage of life history, the testis might accelerate the rate of age-related functional decline or contribute to the development of degenerative diseases in essential organs, such as the brain.

For more than a half century, urologists and oncologists have attempted to manipulate the endocrinologic milieu of men suffering from prostate cancer. Suppression of testicular androgens has been considered the cornerstone of the treatment for advanced prostate cancer since the original report by Huggins and Hodges in 1941 [34]. With the recognition that adrenal steroids contribute a potentially significant androgenic stimulus to the aging prostate and the importance of prostatic intracrinology [35], androgen-deprivation therapy has progressed to an increased level of sophistication. An unanswered question is whether the expanding arsenal of hormonal therapies, initially developed for the treatment of prostate cancer, might also have the potential to exert beneficial effects on essential organs, which might in turn improve quality of life and longevity. If so, interventions to antagonize the testis would have much broader therapeutic application than the treatment of prostate cancer.

Should the testes go or stay? Considering the concept of antagonistic pleiotropy and the observations presented in this paper, the timing and tissue-specificity of orchiectomy or any other endocrine manipulation seems to be important. The consequences of any intervention designed to prolong the human health span by modulating the hormonal milieu will be highly dependent on the timing of the intervention. However, many unanswered questions remain. For example, if the testis adversely affects longevity, will orchiectomy at age 50 or 70 years have similar life-extending effects? Presently, the pharmaceutical industry is placing high priority on the design of tissue-specific endocrine interventions [36]. This makes good sense, because significantly altering the hormonal milieu of the prostate and brain of elderly men may be indicated, whereas it may be desirable to preserve (or perhaps even augment) the effects of the testis on musculoskeletal tissues. Ultimately, it may be possible to intelligently design endocrine interventions that can delay or prevent the onset of important age-related diseases, such as cancer, and cardiovascular or neurodegenerative diseases. Further research to determine if and by what mechanisms the testis plays an important role in these diseases seems prudent.

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SELENIUM AND PROSTATE CANCER: STUDIES USING THE SPONTANEOUS DOG MODEL OF PROSTATE CARCINOGENESIS

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INTRODUCTION

Prostate cancer is responsible for the death of approximately 40,000 men annually in the United States. Advances in prevention rather than treatment may hold the most promise for decreasing the high morbidity and mortality associated with this disease. Successful chemoprevention of prostate cancer will rely upon identification of non-toxic compounds that interfere with key steps in cancer development and progression. Widespread screening of potential chemopreventive agents in humans is impractical; such studies are cost prohibitive and cannot be completed in a timely manner. Currently, the *in vivo* screening of chemopreventive agents in animals relies largely upon rodent models using carcinogen exposures that do not parallel human exposure. A practical, spontaneous model of prostate carcinogenesis could overcome these shortcomings. The dog is the only non-human species in which high-grade prostatic intraepithelial neoplasia (HGPIN) and prostate cancer occur spontaneously (1,2). Although clinically apparent prostate cancer occurs sporadically in pet dogs, we hypothesized that the early events in prostate carcinogenesis may be occurring in the dog prostate with high frequency. This hypothesis was supported by our finding that HGPIN, the putative precursor of many human prostate cancers, is present in 55% of totally embedded, step-sectioned prostates from elderly, sexually intact male pet dogs (3). Our data from dogs provided additional evidence that HGPIN is a consistent step in the progression of prostate cancer because: (1) canine HGPIN is strongly associated with invasive carcinoma; (2) canine HGPIN bears remarkable morphologic similarity to its human counterpart; and (3) basal cell layer integrity, microvessel density, and proliferative index of canine HGPIN are intermediate between normal epithelium and invasive carcinoma (4). The importance of HGPIN as a surrogate endpoint biomarker for chemopreventive studies in humans has been established (5) and clinical trials have been initiated.

Recent interest has focused on the possibility that the essential nutrient, selenium, may significantly influence the risk for prostate cancer development. In a randomized, placebo-controlled clinical trial in patients with a history of skin cancer, selenium supplementation (200 μg per os daily) had no significant effect on the incidence of skin cancer (6). However, the selenium supplemented group experienced a 63% reduction in prostate cancer incidence as well as a marked reduction in overall cancer incidence and mortality. In another study, prediagnostic selenium levels in toenails were inversely correlated with the risk for development of advanced prostate cancer (7). Men with the highest toenail selenium concentration had significantly reduced risk of prostate cancer development [OR + 95% confidence interval = 0.49 (0.25 – 0.96)] compared to men with the lowest selenium levels. The precise mechanism by which selenium modulates the process of prostate carcinogenesis is not clear, but may involve: selenium-dependent antioxidant enzymes; effects on carcinogen metabolism, DNA damage or repair; or modulation of the immune system. The role of selenium as an anticarcinogen has recently been reviewed (8).

In comparison to other human cancers, prostate carcinoma is the malignancy most strongly associated with aging (9). Our work has shown that the development of prostate cancer in pet dogs and humans is similarly affected by aging (10). We and others (11) hypothesize that the accumulation of oxidative DNA damage may provide a mechanistic explanation for the strong association between prostate cancer and aging.

We propose that the canine model is ideally suited for the study of prostate carcinogenesis and in particular the *in vivo* screening of putative chemopreventive agents. At present, we are conducting a six-month trial in elderly male beagle dogs to determine if selenium supplementation favorably modulates biomarkers of DNA damage or prostate carcinogenesis. In this paper, we present our preliminary data on the effect of selenium supplementation on one of these biomarkers – basal level of DNA damage in peripheral blood lymphocytes.

METHODS

Elderly (8 to 10 year-old) sexually intact male beagle dogs were randomly assigned to groups: no treatment (control group; n=5 dogs), 3μg/kg SelenoPrecise™ High Selenium Yeast (Cypress Systems, Inc., Fresno, CA) (low dose group; n=5 dogs), and 6μg/kg SelenoPrecise™ (high dose group; n=4 dogs). After five months of treatment, single cell gel electrophoresis (comet assay) was used to measure basal levels of DNA damage in peripheral blood lymphocytes. The alkaline comet assay is a robust and versatile assay which has been used to measure DNA damage in a wide variety of normal and transformed cells (12). The comet assay was performed using the procedure modified from the method of Singh (13). All experiments used freshly harvested peripheral blood lymphocytes, 60 minutes alkaline unwinding, and 30 minutes electrophoresis (25V, 300mA) at pH > 13. Under these conditions, the assay detects DNA strand breaks, alkali-labile sites, DNA crosslinks, and sites of base excision repair. For each dog, extent of DNA damage was scored in 100 cells using a modification of Collins' method (14). Each cell was scored as follows: Type 0 = no damage, Type 1 & 2 = mild to moderate damage, Type 3 & 4 = extensive DNA damage. Mean percentage of extensively damaged cells (Type 3 & 4) were compared between treatment groups using an ANOVA in SAS (SAS Systems, Cary, NC).

RESULTS

Dogs in the low dose selenium group had significantly reduced basal levels of DNA damage compared to age-matched controls. Mean (± SD) percentage of cells with extensive DNA damage was 25 ± 3% and 11 ± 2% for control dogs and selenium supplemented dogs, respectively (p=0.003). In contrast, the mean percentage of cells with extensively damaged DNA in the high dose selenium group was 28 ± 10%, which was not significantly different from the control group (p=0.31). No adverse side effects were observed in dogs receiving selenium supplementation.

DISCUSSION

Using the alkaline comet assay, we found that elderly male dogs receiving 3μg/kg SelenoPrecise™ had significantly reduced basal levels of DNA damage detectable in their peripheral blood lymphocytes compared to unsupplemented dogs. This 3μg/kg selenium dose is comparable to a human dose of 200μg per day. The influence of selenium supplementation on DNA damage, stromal senescence, and epithelial carcinogenesis within the dog prostate is currently being investigated. These studies have potentially important implications for the field of prostate cancer research.

The results of our ongoing studies will demonstrate the practicality of the dog model for the *in vivo* screening of chemopreventive agents. A long-term objective of this research is to utilize the dog as a preclinical model to test innovative ideas in cancer prevention and treatment, as well as to further understand the mechanisms of prostate cancer development. This research will benefit the health and welfare of both dogs and humans.

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Extent of DNA Damage in Prostate of Elderly Dogs is Abrogated by Selenomethionine Supplementation

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During the next 12 years, the NCI sponsored PCPT-2 SELECT trial will study more than 32,000 men to determine if daily supplementation with selenium ± vitamin E decreases the incidence of prostate cancer. However, the molecular mechanisms by which supranutritional selenium modulates key events in multistep prostate carcinogenesis remains unclear. In this study, we tested the hypothesis that supranutritional selenium exerts a DNA damage-sparing effect on the aged prostate using the dog model. Elderly (8 to 10 year-old) male beagle dogs were randomly assigned to groups: no treatment (sexually intact controls; n=8 dogs), 3μg/kg selenomethionine daily (n=10 dogs), and 6μg/kg selenomethionine daily (n=9 dogs). After seven months of treatment, dogs were euthanatized and single cell gel electrophoresis (alkaline comet assay) was used to measure basal levels of DNA damage in freshly harvested prostate tissue. Mean (± sd) percentage of cells with extensively damaged DNA was compared between treatment groups using ANOVA. Dogs in the low and high dose selenomethionine groups had significantly reduced basal levels of DNA damage compared to age-matched controls. Mean (± sd) percentage of prostate cells with extensive DNA damage was 80 ± 5% in control dogs. In contrast, extensive DNA damage was seen in 58 ± 9% and 62 ± 14% of prostate cells from dogs that received daily supplementation with 3μg/kg or 6μg/kg selenomethionine, respectively ($p<0.01$ vs. controls). This model enables us to study the anticarcinogenic mechanisms of selenium supplementation in a model of selenium adequacy, which is the case in almost all healthy Americans, including those who will participate in the SELECT trial. [Supported in part by a grant from the USAMRMC Prostate Cancer Research Program (PC-970492 awarded to DJW)]

Reduction in DNA Damage in Brain and Peripheral Blood Lymphocytes of Elderly Dogs Supplemented with Dehydroepiandrosterone (DHEA)

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It is likely that DNA damage contributes to organismal senescence and increased risk for specific age-related diseases. Human aging is accompanied by a profound decline in plasma levels of the adrenal steroid, dehydroepiandrosterone (DHEA), and DHEA-S (Orentreich et al, J Clin Endocrinol Metab 1984; 59:551). DHEA exhibits significant cancer chemopreventive activity in rodent models of prostate and breast cancer (McCormick and Rao, Eur Urol 1999; 35:464). Beneficial effects of DHEA supplementation in humans have also been reported (Morales et al, J Clin Endocrinol Metab 1994; 78:1360) and increasing evidence suggests that DHEA is a neuroactive agent (Robel and Baulieu, Ann NY Acad Sci 1995; 774:82). To our knowledge, the influence of DHEA supplementation on DNA damage has not been previously investigated. We studied elderly dogs to determine if supplementation with DHEA significantly reduces basal levels of DNA damage in the brain or peripheral blood lymphocytes. Elderly (8 to 10 year-old) male beagle dogs were randomly assigned to groups: no treatment (sexually intact controls; n=4 dogs), and DHEA (Genelabs Technologies, Inc., Redwood City, CA) 100mg/kg supplementation daily (n=4 dogs). Six months later, single cell gel electrophoresis (comet assay) was used to measure levels of DNA damage in brain and peripheral blood lymphocytes. Mean (± sd) percentage of cells with extensively damaged DNA was compared between treatment groups. Analysis of brain tissue from DHEA treated dogs showed that 53 ± 7% cells had extensive DNA damage. This was significantly less than the DNA damage in brain tissue of control dogs, which showed 82 ± 8% of cells had extensively damaged DNA ($p=0.0017$). The DHEA supplemented group also had a significantly reduced percentage of peripheral blood lymphocytes with extensive DNA damage (mean ± sd = 9 ± 1% compared to 24 ± 2% in control group; $p<0.0001$). These preliminary data suggest that DHEA supplementation can significantly reduce the accumulation of DNA damage in the mammalian brain. Further analysis of the effects of DHEA supplementation on DNA damage and gene expression in specific tissues is warranted. [Supported in part by a grant from the USAMRMC Prostate Cancer Research Program (PC-970492 awarded to DJW)]

THE TESTIS CONTRIBUTES TO THE ACCUMULATION OF DNA DAMAGE IN THE AGING MAMMALIAN BRAIN

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The disparity in life expectancy between men and women is a well recognized phenomenon in most countries of the world. The theory of antagonistic pleiotropy, proposed over 40 years ago[1], predicts certain genes that are beneficial early in life exert detrimental effects during the post-reproductive senescent period. We hypothesized that the testis, which is essential early in life for reproductive success, may diminish life expectancy through detrimental effects on cellular processes in essential organs, such as the brain. To test this hypothesis, we studied dogs to determine if castration reduces the extent of DNA damage within the brain. Eight, sexually-intact male beagle dogs 9 to 10.5 years of age (62 to 69 years in human age equivalents[2]) were randomly assigned to receive either no treatment or surgical castration. Six months later, the extent of nuclear DNA damage (single strand breaks, alkali-labile sites, and sites of base excision repair) was measured in brain tissue and peripheral blood lymphocytes using the alkaline comet assay[3]. All experiments used freshly harvested brain tissue (cerebral cortex) or peripheral blood lymphocytes, 20 minutes alkaline unwinding, and 30 minutes of electrophoresis (25V, 300mA) at pH>13. For each dog, extent of DNA damage was scored per 100 cells using a modification of Collins' method[4]. Each cell was scored as follows: Type 0 = no damage; Type 1 & 2 = mild to moderate damage; Type 3 & 4 = extensive DNA damage. Mean (± standard deviation) percentage of extensively damaged cells (Type 3 & 4) were compared between treatment groups using an ANOVA. Thirty-seven ± 18 percent of cells in the cerebral cortex of castrated dogs had extensively damaged DNA, compared with 83 ± 8 percent of cells in the control group ($p=0.0005$). The castrated group also had a two-fold reduction in the percentage of peripheral blood lymphocytes with extensive DNA damage (mean ± SD = 12 ± 2 compared to 24 ± 2 in the control group; $p<0.0001$). These data provide the first evidence that the testis contributes to the accumulation of DNA damage within the aging mammalian brain. Accumulation of DNA damage has been implicated in age-related functional decline in the human brain[5,6]. It is widely accepted that testicular androgens have undesirable effects late in life by stimulating the development of prostatic disease. However, our findings suggest that the aging brain, a previously unrecognized target, may also be vulnerable to the potentially adverse effects of the testis. The expanding arsenal of endocrine manipulations, initially developed for the treatment of prostate cancer, might have the capacity to decelerate age-related degeneration within essential organs, such as the brain. If so, interventions to antagonize the testis might have broader clinical application than previously expected.

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REDUCTION IN DNA DAMAGE IN BRAIN AND PERIPHERAL BLOOD LYMPHOCYTES OF ELDERLY DOGS SUPPLEMENTED WITH DHEA

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Elderly (8 to 10 year-old) male beagle dogs were randomly assigned to groups: no treatment (sexually intact controls; n=4 dogs), and DHEA (Genelabs Technologies, Inc., Redwood City, CA) 100mg/kg supplementation daily (n=4 dogs). Six months later, single cell gel electrophoresis (comet assay) was used to measure levels of DNA damage in brain and peripheral blood lymphocytes. Mean ($\pm$ sd) percentage of cells with extensively damaged DNA was compared between treatment groups. Analysis of brain tissue from DHEA treated dogs showed that $53 \pm 7\%$ cells had extensive DNA damage. This was significantly less than the DNA damage in brain tissue of control dogs, which showed $82 \pm 8\%$ of cells had extensively damaged DNA ($p=0.0017$) The DHEA supplemented group also had a significantly reduced percentage of peripheral blood lymphocytes with extensive DNA damage (mean $\pm$ sd = $9 \pm 1\%$ compared to $24 \pm 2\%$ in control group; $p< 0.0001$). Further analysis of the effects of DHEA supplementation on DNA damage and gene expression in specific tissues is warranted.

REDUCTION IN DNA DAMAGE IN PERIPHERAL BLOOD LYMPHOCYTES OF ELDERLY MALE DOGS RECEIVING DHEA SUPPLEMENTATION OR ORCHIECTOMY

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It is likely that DNA damage contributes to at least two age-related processes: organismal senescence and cancer development. To our knowledge, the influence of DHEA supplementation or androgen ablation on host DNA damage has not been previously investigated. The purpose of this study was to determine if orchiectomy or supplementation with DHEA significantly reduces basal levels of DNA damage in elderly dogs. Elderly (8 to 10 year-old) intact male beagle dogs were randomly assigned to groups: no treatment (sexually intact controls; n=5 dogs), DHEA (Genelabs Technologies, Inc., Redwood City, CA) 100mg/kg supplementation daily (n=5 dogs), and orchiectomy (n=4 dogs). Five months later, single cell gel electrophoresis (Comet assay) was used to measure basal levels of DNA damage in peripheral blood lymphocytes. Extent of DNA damage was scored in 100 cells as follows: Type 0 = no damage, Type 1 & 2 = mild to moderate damage, Type 3 & 4 = extensive DNA damage. Mean percentage of extensively damaged cells (Type 3 & 4) were compared between treatment groups. Mean ± SD percentage of cells with extensive DNA damage was 24.2±2.2%, 16.5±3.3%, and 8.0±1.4% for intact controls, orchiectomized dogs and DHEA supplemented dogs, respectively. The three-fold difference in basal DNA damage in the DHEA supplementation group compared to the intact control group was highly significant ($p<0.0001$). The 33% reduction in basal DNA damage in the orchiectomized group compared to the intact control group was also significant ($p=0.0002$). The influence of these hormonal alterations on DNA damage, stromal senescence and epithelial carcinogenesis within the dog prostate is currently being investigated.

Dose-dependent Reduction in DNA Damage in Peripheral Blood Lymphocytes of Dogs Supplemented with Dietary Selenium

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It is likely that DNA damage contributes to at least two age-related processes: organismal senescence and cancer development. The purpose of this study was to determine if selenium supplementation would significantly reduce DNA damage in elderly dogs. Elderly (8 to 10 year-old) sexually intact male beagle dogs were randomly assigned to groups: no treatment (control group; n=5 dogs), 3μg/kg SelenoPrecise™ High Selenium Yeast (low dose group; n=5 dogs), and 6μg/kg SelenoPrecise™ (high dose group; n=4 dogs). After five months of treatment, single cell gel electrophoresis (Comet assay) was used to measure basal DNA damage in peripheral blood lymphocytes. In all experiments, freshly harvested peripheral blood lymphocytes and 30 minute electrophoresis at pH > 13 were used. Extent of DNA damage was scored in 100 cells using a modification of Collins' method. Each cell was scored as follows: Type 0 = no damage, Type 1 & 2 = mild to moderate damage, Type 3 & 4 = extensive DNA damage. Mean percentage of extensively damaged cells (Type 3 & 4) were compared between treatment groups. Dogs in the low dose selenium group had significantly reduced basal DNA damage compared to age-matched controls. Mean (± SD) percentage of cells with extensive DNA damage was 25 ± 3% and 12 ± 2% for control dogs and selenium supplemented dogs, respectively ($p<0.01$). In contrast, the mean percentage of cells with extensively damaged DNA in the high dose selenium group was 28 ± 12%, which was not significantly different from the control group. In conclusion, using the Comet assay, we found that elderly male dogs receiving 3μg/kg SelenoPrecise™ had significantly reduced basal DNA damage detectable in their peripheral blood lymphocytes compared to unsupplemented dogs. The influence of selenium supplementation on DNA damage, stromal senescence, and epithelial carcinogenesis within the dog prostate is currently being investigated.